

Integrated Control of Fire Blight with Antagonists and Oxytetracycline

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Abstract

In the Northwest United States, the antibiotic streptomycin provided excellent control of fire blight until resistant isolates of the pathogen arose. Oxytetracycline (Mycoshield) is now sprayed as an alternative antibiotic. We found that the durability of inhibitory activity of oxytetracycline is similar to that of streptomycin, but oxytetracycline is considerably less effective than streptomycin when the antibiotics are targeted toward sensitive strains. In an effort to improve disease control, we evaluated combinations of biological control agents (*Pseudomonas fluorescens* A506 or *Pantoea agglomerans* C9-1S) and oxytetracycline in eight orchard trials inoculated with an antibiotic-sensitive strain of *Erwinia amylovora*. Two bloom sprays of streptomycin or oxytetracycline reduced the disease incidence by an average of 76% and 42%, respectively, compared to water-treated controls. A combination of C9-1 and a protease-deficient A506 provided 42% disease control. An integrated treatment, i.e., a spray of biological control agents followed by one application of oxytetracycline provided 57% control. Biological and chemical methods of fire blight suppression appear to be complementary, and consequently, an integrated strategy consisting of a biological control agent sprayed in early and near full-bloom, followed by oxytetracycline treatment at late bloom improved disease control with a reduced number of antibiotic applications.

INTRODUCTION

In the United States, the antibiotic oxytetracycline (Mycoshield, NuFarm Americas, Burr Ridge, IL) is registered to manage fire blight on pear. The antibiotic is used especially in regions where streptomycin-resistant isolates of the pathogen *Erwinia amylovora* are common (Loper et al., 1991; McManus et al., 2002). Oxytetracycline is sprayed onto trees during bloom at concentrations of 100 to 200 µg/ml. No isolates of *E. amylovora* resistant to oxytetracycline at the concentrations applied to trees have been detected. As a stand-alone treatment, products containing oxytetracycline reduce the incidence of fire blight by about 40%, which is about half the level of suppression obtained by streptomycin in orchards inoculated with an antibiotic-sensitive pathogen. The relative persistence of antibiotic activity of both compounds on flowers is not known but was postulated that the duration of inhibitory activity of oxytetracycline on flowers was less than streptomycin (McManus et al., 2002). Vanneste (1996) demonstrated that streptomycin controls fire blight when the pathogen is applied up to 5 days after a streptomycin spray. One goal of this study was to determine the relative duration of inhibition of commercial antibiotics on flowers.

In addition to antibiotics, three Gram negative bacterial biocontrol agents for fire blight are registered in the US. They are BlightBan A506 (*Pseudomonas fluorescens* strain A506; NuFarm Americas), BlightBan C9-1 (*Pantoea agglomerans* strain C9-1S; NuFarm Americas) and Bloomtime Biological (*P. agglomerans* strain E325; Northwest Agricultural Products, Pasco, WA). Of these, only BlightBan A506 was commercially available prior to 2007. Recently, we derived a protease-deficient deletion mutant of A506 called A506 $\Delta aprX$, which does not inactivate the antibiotic pantocin A produced by C9-1 like the wild-type strain A506 (Anderson et al., 2004). In our field trials, a

combination of C9-1 with A506 $\Delta aprX$ has provided the best control with the least variation in efficacy, compared to single strain inoculants or a mixture of A506 with C9-1. In spite of improved control, mixtures of A506 $\Delta aprX$ and C9-1 still did not consistently approach the level of control of fire blight obtained with streptomycin (unpublished data).

The goal of this research was to evaluate if combining two moderately-effective disease management tools, oxytetracycline and biological control agents, improves control of fire blight. In previous research (Stockwell et al., 1996), oxytetracycline was toxic to C9-1 or A506 in suspension, but the biological control agents established on flowers tolerated oversprays of Mycoshield. In this study, we evaluated the control of fire blight with biological control agents and Mycoshield applied as sequential sprays.

MATERIALS AND METHODS

Duration of Antibiotic Activity on Flowers

Four experiments on the effect of antibiotics on establishment and growth of *E. amylovora* on flowers were conducted on pear and apple trees (7 to 12 years old) located in a screenhouse at the Oregon State University, Department of Botany and Plant Pathology Field Laboratory near Corvallis, OR. Trees in the screenhouse were protected from rain and ultraviolet radiation by a translucent, fiberglass roof and from insects by 2 x 2 mm steel screen walls. In 2004 and 2005, newly opened flowers of three replicate trees per treatment of pear (*Pyrus communis* cv. Bartlett) and crabapple (*Malus* × 'Snowdrift') were sprayed to near run-off with Agri-mycin 17 (100 µg per ml, streptomycin sulfate 17% a.i., NuFarm Americas), Mycoshield (200 µg/ml, oxytetracycline calcium complex, 17% a.i., NuFarm Americas) or a mixture of the two antibiotics. Control trees were sprayed with water. On 1, 2, 4, 6 and 9 days after spraying, 30 flowers per tree were marked with colored wires tied around the petiole, and the stigmas of each flower were inoculated by pipetting 5 µl of a 5×10^5 CFU/ml suspension of lyophilized cells of *E. amylovora* strain 153N; the applied dose of the pathogen was 1×10^3 CFU per flower. The pathogenic strain Ea153N is sensitive to streptomycin and oxytetracycline but resistant to nalidixic acid. One day after each inoculation and every two to three days thereafter, 5 flowers per replicate tree (15 per treatment) were harvested. The pistils from each flower were placed in 1 ml sterile 10 mM phosphate buffer, sonicated for 3 min, vortexed, and 10 µl of the flower wash and two 100-fold dilutions were spread on CCT medium amended with nalidixic acid (50 µg/ml) to enumerate the pathogen Ea153N (Ishimaru et al., 1984). After three days incubation, colonies were counted and converted into CFU per flower. A value of 99 CFU (the detection limit minus 1) was assigned to samples with populations of Ea153N below the detection limit.

Efficacy of Integrated Control in Experimental Orchards

Experimental pear and apple orchards were located at the OSU Botany and Plant Pathology Field Laboratory near Corvallis, OR. The mature orchards were 0.5 ha blocks of pear 'Bartlett' and of apple 'Golden Delicious' and 'Rome Beauty.' The experiments were arranged in a randomized, complete block design with 4 to 5 replications and 5 or 7 treatments applied to single trees. Blossom cluster density and tree location were considered in the assignment of individual trees to blocks in the plot design. The antibiotic Agri-mycin 17 and water were included as standard controls.

Mycoshield was applied alone or as an overspray on trees treated with mixtures of biological agents. The biocontrol mixtures consisted of BlightBan C9-1 (*Pantoea agglomerans* C9-1S a.i., NuFarm Americas) mixed with BlightBan A506 (*Pseudomonas fluorescens* A506 a.i., NuFarm Americas) or lyophilized cells of the experimental protease-deficient deletion mutant of A506 called A506 $\Delta aprX$.

All treatments were sprayed at sunrise to near run-off to trees in 70% bloom with a 12 L backpack sprayer equipped with a hand wand. The antibiotic treatments also were sprayed within 2 to 3 days after inoculation with the pathogen. Trees were inoculated with

Ea153N by misting a suspension containing 5×10^5 CFU per ml of freeze-dried cells of the pathogen with a motorized, 100 L tank sprayer equipped with a hand-held adjustable brass nozzle.

Periodically, 8 flowers were sampled from each tree to enumerate populations of the biological control agents and the pathogen. The pistils (apple) and pistils and nectary (pear) were removed from each flower, placed into 1 ml of sterile phosphate buffer and sonicated for 3 min. After vortexing, a 10- μ l sample of the flower wash and two 1:100 dilutions were spread on *Pseudomonas* agar F with rifampicin (50 μ g/ml) and cycloheximide (50 μ g/ml) to enumerate biological control agents and on CCT medium with nalidixic acid (50 μ g/ml) to enumerate Ea153N.

Incidence of fire blight was determined by counting the number of blighted blossom clusters (i.e., strikes) on each tree during weekly inspections of pear from mid-April to mid-May, and of apple from mid-May through June. Blighted blossom clusters were removed immediately after counting. The sum of blighted blossom clusters per tree was converted into disease incidence (total diseased clusters/total number of clusters per tree) which was arcsine-square root transformed and subjected to analysis of variance.

RESULTS AND DISCUSSION

Duration of Antibiotic Activity

In each trial, the commercial formulations of the antibiotics streptomycin and oxytetracycline inhibited growth of *E. amylovora* pipetted onto floral stigmas one and two days after antibiotic application (Fig. 1A, B). The results from application of Agri-mycin 17 combined with Mycoshield were indistinguishable from the effect of Agri-mycin 17 alone on flowers (data not shown). On flowers inoculated one day after antibiotic spray, both compounds inhibited growth for six days. The pathogen multiplied on flowers treated with Mycoshield after that time but only a slight population increase was observed on flowers treated with Agri-mycin 17 (Fig. 1A). The incidence of detectable populations of Ea153N differed on flowers treated with the antibiotics. For each trial, about 25% of the flowers inoculated with Ea153N within two days after Agri-mycin 17 treatment had detectable populations of the pathogen. In contrast, Ea153N was detected on 87 to 100% of flowers inoculated within two days after Mycoshield treatment. The greater incidence of recovery of Ea153N on flowers treated with Mycoshield compared to the incidence on Agri-mycin 17 treated flowers is expected because oxytetracycline is bacteriostatic, not bactericidal like streptomycin (McManus and Jones, 1994).

When Ea153N was applied four days after antibiotic sprays, the pathogen attained similar population sizes within six days after inoculation as on water-treated flowers (Fig. 1C). The pathogen was recovered from nearly every flower inoculated after 4 days of spraying with water or Mycoshield. Ea153N was detected on about 75% of flowers sprayed with Agri-mycin 17 four days before inoculation. Similar results were obtained with flowers inoculated 6 days after antibiotic treatment (data not shown).

Ea153N grew poorly when inoculated on flowers at petal fall or nine days after water or antibiotic treatment (Fig. 1D). Growth of the pathogen at this time was similar among flowers treated with antibiotics or water. The relatively poor growth of Ea153N on flowers inoculated during petal fall was likely due to floral age, confirming previous observations of Thomson and Gouk (2003).

We conclude that the inhibitory activity of streptomycin and oxytetracycline on flowers under rain-free conditions is about four days, which is similar to the results on streptomycin by Vanneste (1996). Low populations of Ea153N survived on a high percentage of flowers sprayed previously with Mycoshield, whereas few flowers treated with Agri-mycin 17 supported populations of Ea153N. The bactericidal activity of streptomycin likely explains the better disease control with this antibiotic compared to oxytetracycline; not differences in duration of the active antibiotics on flowers.

Influence of Integrated Control Methods on Populations of Biological Control Agents and the Pathogen

Biological control agents established on flowers survived a subsequent spray of Mycoshield, although the incidence of recovery and the mean population size of each bacterium were depressed compared to populations on flowers not oversprayed with oxytetracycline. Generally the mean population size of the biological control agents on flowers sprayed with Mycoshield was 1 log unit lower and the incidence of recovery was decreased to 75% of flowers sampled, compared to 80 to 90% incidence of recovery of the biocontrol agents on flowers not treated with Mycoshield.

The population size of the pathogen was generally lower on flowers treated with biological control agents followed by a single application of oxytetracycline compared to flowers treated with only biological control agents, water or two applications of oxytetracycline, but the magnitude of population suppression varied among trials. For example, the incidence of recovery of Ea153N on water treated flowers was 94% with a mean population size of log 5.8 on 'Golden Delicious' apple in 2007, nine days after pathogen inoculation. Ea153N was recovered from 53 and 69% of flowers with a mean population size of log 4.8 and 4.4 on trees treated with A506 and C9-1 combined and Mycoshield alone, respectively. On flowers treated with A506 & C9-1 followed by Mycoshield, Ea153N was recovered at a mean population size of log 4.5 on 44% of the flowers.

Greater suppression of the pathogen with the integrated control strategy was observed on Bartlett pear in 2007. Eight days after inoculation, the pathogen was recovered from 100% of flowers on water-treated control trees at a mean population size of log 5.5. Ea153N was recovered from 84 and 44% of flowers with a mean population size of log 5.2 and 3.8 on trees treated with only A506 and C9-1 or Mycoshield, respectively. On flowers treated with A506 and C9-1 followed by Mycoshield, Ea153N was recovered from only 9% of flowers at a mean population size of log 2.6.

Efficacy of Integrated Disease Control

In orchard trials conducted in 2005, 2006 and 2007 on pear and apple, Agri-mycin 17 provided better control of fire blight than Mycoshield when trees were inoculated with a strain of the pathogen sensitive to both antibiotics (Table 1). The mixture of the biological control agents A506 $\Delta aprX$ and BlightBan C9-1 provided better control of fire blight (42% reduction in disease incidence) than BlightBan A506 and BlightBan C9-1 (17% reduction in disease incidence), but the difference in control was not significant among individual trials. The level of control in this study with the mixture of C9-1 and A506 was less than that reported by Johnson et al. (1993), which may be due to the experimental methods used. Johnson et al. (1993) applied the biocontrol strains twice and inoculated trees with bee-dispersed lyophilized cells of the pathogen; whereas in this study the biocontrol agents were applied only once and trees were inoculated uniformly by misting trees with suspensions of lyophilized cells of the pathogen. The reduced number of applications of the biocontrol agents coupled with the uniform application of the pathogen may have resulted in increased disease pressure and reduced efficacy of biological control in the current study.

The integrated strategy of applying biological control agents once followed by a single application of Mycoshield provided better control than biological control agents alone (Table 1). A506 and C9-1 reduced disease by 17%; whereas a single overspray with Mycoshield improved disease control to 49%. A506 $\Delta aprX$ and C9-1 provided 42% control and adding one overspray of Mycoshield improved disease control to 57%. The integrated control strategy often provided better control than the conventional method of spraying Mycoshield twice, which had 42% disease control. Two applications of Agri-mycin 17 gave 76% disease control, which was not statistically different than a single treatment with A506 $\Delta aprX$ and C9-1 followed by a single application of Mycoshield.

CONCLUSIONS

The integrated control strategy may reduce the number of antibiotic sprays applied to pear and apple trees, while still providing disease control. Ninety percent of the amount of antibiotics used on plants in the US is sprayed on pear and apple trees for fire blight management (McManus et al., 2002). Reduction in the number of antibiotic sprays for fire blight control may reduce the selection pressure for development of antibiotic-resistant isolates of *E. amylovora* and also reduce exposure of the environment and orchard workers to antibiotics.

We evaluated Mycoshield (oxytetracycline), which is a moderately effective antibiotic for fire blight management, as the chemical component of the integrated control strategy. The improved control with biocontrols oversprayed with Mycoshield is likely due to several factors. Generally, we found that treatment of flowers with biological control agents and Mycoshield did not kill the fire blight pathogen, but this two-pronged approach hampered the growth or establishment of the pathogen on flowers. We speculate that the reduction in growth rate or establishment of the pathogen may allow flowers to progress through their natural developmental stages from highly susceptible to less susceptible to infection before the pathogen attains high population sizes (Thomson and Gouk, 2003).

In the future, the use of other chemical components that are toxic to the pathogen with biological controls may improve fire blight control further. For example, Lindow et al. (1996) combined streptomycin with A506 and significantly improved fire blight control compared to the single components. Johnson et al. (2008) found that kasugamycin provided very good disease control, but he cautioned that the antibiotic adversely affected populations of C9-1 and A506 $\Delta aprX$ when used as an integrated program. The use of bactericidal compound(s) to which the pathogen is sensitive and biocontrol agents are resistant will likely significantly improve integrated control strategies for fire blight.

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Tables

Table 1. Relative control of fire blight in experimental orchards in Oregon.

Treatment ^a	Trial (year and cultivar)					
	2005			2006		
	Bartlett	Golden Delicious	Rome Beauty	Bartlett	Golden Delicious	Rome Beauty
Water	0 ^b A	0 A	0 A	0 AB	0 A	0 AB
A506 & C9-1	[66] ^c	[296]	[19]	[162]	[1622]	[230]
A506 & C9-1 then Mycoshield	NT ^d	NT	NT	-11 A	6 A	0 AB
A506 $\Delta aprX$ & C9-1	NT	NT	NT	46 CD	48 B	6 AB
A506 $\Delta aprX$ & C9-1 then Mycoshield ^{ad}	54 B	65 AB	44 AB	10 ABC	33 AB	23 B
Mycoshield	79 C	77 BC	56 BC	57 DE	33 AB	36 B
Agri-mycin 17	79 C	42 AB	41 AB	26 ABCD	33 AB	-1 A
	93 C	75 B	83 C	82 E	33 AB	75 C

^aAll trees in experimental orchards were inoculated during full bloom with 5×10^5 to 1×10^6 CFU/ml *Erwinia amylovora* strain Ea153N (streptomycin- and oxytetracycline-sensitive fire blight pathogen strain). Biological control bacteria *Pantoea agglomerans* C9-1 and *Pseudomonas fluorescens* A506 were applied once as the commercial formulations called BlightBan at 5×10^7 CFU/ml for each strain. Resuspended freeze-dried cells of the protease-deficient mutant of A506 called A506 $\Delta aprX$ was substituted for A506 in mixtures and applied at 5×10^7 CFU/ml. Water and antibiotics [Mycoshield (a.i. oxytetracycline, 200 ppm) and Agri-mycin 17 (a.i. streptomycin, 100 ppm)] were applied at 80% bloom and ca. 36 h after inoculation of trees in full bloom with the pathogen. For the integrated treatments, biological control agents were sprayed once at 80% bloom and Mycoshield was sprayed once after full bloom.

^bRelative disease control presented as mean reduction in disease incidence. The incidence of disease on water-treated and inoculated trees was set at 100%. Disease control for treatments was calculated as percent decrease in disease incidence relative to water treatment. Values followed by the same letter within a column containing data from a single orchard trial are not significantly different according to Fischer's protected least significance difference at $P=0.05$. Data were transformed arcsine (square root(x)) prior to analysis.

^cNumbers in parentheses are the average number of strikes (blossom clusters with symptoms of fire blight) on water-treated and inoculated trees.

^dNT indicates treatment not tested in that trial.

Figures

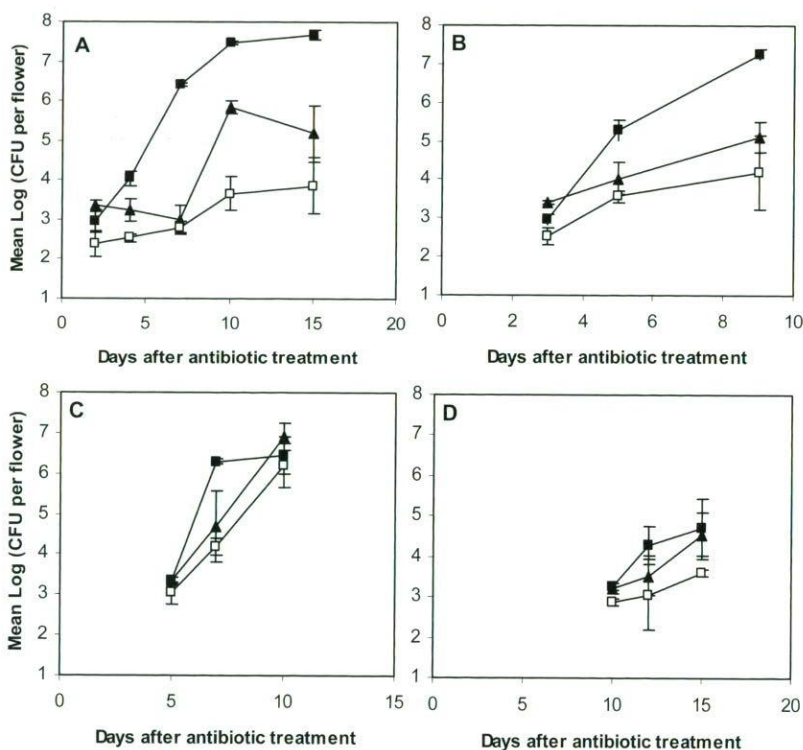


Fig. 1. Mean population size (\log_{10} CFU per flower) of *Erwinia amylovora* strain 153N on 'Bartlett' pear flowers sprayed with water (■), Agri-mycin 17 (□, 100 $\mu\text{g/ml}$ streptomycin sulfate) or Mycoshield (▲, 200 $\mu\text{g/ml}$ oxytetracycline calcium) and then periodically inoculated with the pathogen. A) Populations of Ea153N on flowers inoculated 1 day after water or antibiotic sprays. B) Populations on flowers inoculated 2 days after sprays. C) Populations on flowers inoculated 4 days after sprays. D) Populations on flowers inoculated 9 days after sprays. Each point represents the mean population size and vertical bars represent \pm one standard error.